Departement für Nutztiere, Klinik für Reproduktionsmedizin

der Vetsuisse-Fakultät Universität Zürich

Direktor: Prof. Dr. H. Bollwein

Populationsgenetische Untersuchung ektopischer Ureteren beim Entlebucher Sennenhund

Inaugural-Dissertation

zur Erlangung der Doktorwürde der

Vetsuisse-Fakultät Universität Zürich

vorgelegt von

Reto Fritsche

Tierarzt

von Appenzell AI, Schweiz

genehmigt auf Antrag von

PD Dr. Iris Margaret Reichler, Referentin

PD Dr. Claude Schelling, Korreferent

Zürich 2012

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Inhaltsverzeichnis

Abstrakt

Untersuchungsziel— Zur Abklärung der Hypothese einer genetischen Aetiologie von ureteraler Ektopie (UE) wurden beim Entlebucher Sennenhund (ES) verschiedene Risikofaktoren, die mit der Erkrankung assoziiert sind untersucht sowie der Erbgang analysiert.

Design— Multizentrische Querschnittsstudie.

Tiere— 565 privat gehaltene ES.

Methoden— Anhand der weiter kaudal gelegenen ureteralen Mündung wurden 552 ES einer der drei Phänotyp-Gruppen Trigonum, intravesikale und extravesikale Ektopie zugeordnet. Logistische Regression und komplexe Segregationsanalyse wurde zur Analyse eingesetzt.

Ergebnisse— In einem Drittel der untersuchten Hunde mündeten beide Ureteren an der korrekten Stelle, 47% und 20% hatten mindestens einen intravesikulär bzw. extravesikulär ektopisch mündenden Ureter. Als Risikofaktor wurde einzig die männliche Geschlechtszugehörigkeit ermittelt. Inkontinenz, Hydronephrose und/oder Hydroureter waren häufiger mit extravesikulärer Ektopie beim Rüden assoziiert. Nach Zuchtrestriktion wurde eine deutliche Abnahme extravesikulärer UE festgestellt. Die Analyse ergab eine erbliche Genese mit Hinweis auf die Beteiligung eines Hauptgens.

Schlussfolgerung— Die UE des ES ist eindeutig vererbt. Höchstwahrscheinlich sind mehrere Gene für die Fehlbildung verantwortlich. Die Inzidenz-Abnahme der extravesikulären UE nach Zuchtbeschränkung weist auf die Beteiligung eines Hauptgenes hin.

Stichworte— Ureterale Ektopie, Entlebucher Sennenhund, Vererbung , Segregationsanalyse

Abstract

Objectives— In order to test the hypothesis for a heritable basis of ectopic ureters (EUs), risk factors associated with the occurrence and the mode of inheritance of EUs in Entlebucher Mountain Dogs (EMDs) were evaluated.

Design— Multi-center cross-sectional study.

Animals— 565 privately owned EMDs were clinically investigated.

Methods— Based on the location of the most caudal termination of the ureteral opening, 552 EMDs were classified into phenotype groups trigone, intravesically and extravesically ectopic using abdominal sonography, urethra-cystoscopy and/or contrast-enhanced computed tomography. Logistic regression and complex segregation analysis were performed with several datasets resulting from different phenotype groupings.

Results— One third (32.9%) of the animals had normal terminations of both ureters in the bladder trigone, 47.3% had at least one intravesicular ectopic termination and 19.8% had at least one extravesicular ectopic termination. Gender was the only associated risk factor with more males being affected. Incontinence, hydronephrosis and/or hydroureter occurred more often with extravesicular ectopia and also more often in males. A rapid decline of extravesically affected EMDs was observed after kennel clubs established breeding restrictions. The complex segregation analysis rejected the environmental model in all but one dataset. In three datasets no further differentiation between the mixed inheritance, major gene and polygene models was possible. The major gene model was the best fit in one dataset.

Conclusions and Clinical Relevance— EUs in the EMD clearly have a hereditary basis. Most likely several genes are involved. The incidence decline of extravesically affected EMDs suggests the presence of at least one major gene.

Keywords— Ureteral ectopia, Entlebucher Mountain Dog, inheritance, segregation analysis

Abbreviations

EMD Entlebucher Mountain Dog
EU Ectopic ureter
EVEU Extravesicular ectopic ureter
IVEU Intravesicular ectopic ureter
PAP Pedigree analysis package

Introduction

Ureteral ectopia is a congenital abnormality in which one or both ureters terminate in a position other than the trigone of the urinary bladder.[1] Ectopic ureters (EUs) are classified according to the location of their termination as intravesicular (IVEU) or extravesicular (EVEU),[2] and according to their course until their terminal orifice as intramural or extramural.[3]

Intramural EUs, which are reported to be predominant in dogs,[4] enter the bladder wall in the area of the trigone but continue for some distance within the submucosa before opening into the lower portion of the urogenital system.[5] Extramural EUs completely bypass the body of the bladder and end up entering the bladder neck, urethra, vagina, vestibule, uterus or the ductus deferens.[3,6,7]

In the dog, the condition is generally rare with reported incidences below 0.05% for clinically apparent cases.[8-10] The most common clinical sign is the occurrence of urinary incontinence at a young age[11] however, some animals, and especially males, do not show clinical symptoms until they reach an advanced age.[12] Besides incontinence, recurrent urinary tract infections (e.g. cystitis, pyelonephritis), hydroureter and hydronephrosis are of clinical importance.[6,13] In severely affected dogs, the course of the disease may be fatal. Although surgery has a good prognosis for survival if performed early enough, focus should primarily be on the prevention of EUs because of the substantial suffering this disease causes and the high rate of complications after surgery.[14]

Entlebucher Mountain Dogs (EMDs), Briards, Bulldogs, Golden Retrievers, Labrador Retrievers, Griffons, Border Terriers, Fox Terriers, Skye Terriers, West Highland White Terriers, Siberian Huskies, Newfoundland dogs, Miniature and Toy Poodles have been found to be at an increased risk for EUs.[a,b,2,15,16] Genetic involvement was first proposed by Hayes in 1974 after finding certain breeds at high risk for EUs.[9] This hypothesis is supported by case reports describing familial aggregations of ectopic ureters in Siberian Huskies, Golden and Labrador Retrievers, EMDs[b,2,17,18] and Shelties (unpublished data). Familial increased incidence of EUs have also been reported in humans.[19,20]

The fact that EMD puppies died of uremia due to hydronephrosis, or showed urinary incontinence right after birth, together with the suspicion of a breed predisposition for ectopic ureters,[b,14] led to the implementation of a screening program for EUs with the participation of the Swiss, German, Dutch and Austrian EMD kennel clubs (SKES, SSV-ES, ESC and VSSÖ, respectively), evaluating prevalence and clinical relevance.[c] Starting in mid-2008, the Swiss and German kennel clubs required breeding dogs to be examined for presence of EUs. A restrictive breeding program which excluded severely affected dogs was established in 2009. However, due to the high incidence of EUs in the examined EMDs (45% IVEUs and 19% EVEUs)[a] and the small population (effective population size in the breed homeland of less than 50),[e] setting up a reasonable breeding strategy is rather difficult. For selection programs to be successful in minimizing the incidence of undesirable characteristics or diseases and maintaining general health of the breed without increasing inbreeding, knowledge of its heritability and inheritance pattern is crucial. Furthermore, knowledge of the mode of inheritance improves the chance of success for gene mapping, localization and gene identification.[21]

In order to test our hypothesis for a heritable basis of EUs, the objective of this study was to evaluate risk factors associated with the occurrence of EUs and to assess its mode of inheritance in EMDs.

Materials and Methods

Data— In this multi-center, cross-sectional study with subsequent genetic analysis, the initial dataset collected by our group (308 EMDs)[c] was re-evaluated. By including more EMDs and further phenotyping we achieved a better family structure. This screening study of privately owned and kennel club registered EMDs was approved by the Swiss Federal Veterinary Office. Six institutes with board certified radiologists or internists in Switzerland, Germany, Austria and the Netherlands participated in the study. Pedigree information was obtained from FCI accredited pedigree certificates, and every dog was identified by microchip serial number. For each of the examined EMDs the following parameters were recorded on initial presentation: Age, sex and neuter status, country of origin and kennel club. Information on litter size, gender distribution within the litters and number of early-death puppies (stillborn puppies and neonates up to 8 weeks postpartum), was gathered from kennel clubs of Swiss and German EMDs.

Owners were questioned about the occurrence of urinary incontinence, and dogs were clinically examined. Sedation with 0.01mg/kg bodyweight (BW) buprenorphine and 0.015 mg/kg BW acepromazine IM was left to the discretion of the attending clinician depending on the age and the temperament of the patient. An intravenous catheter was placed in the cephalic vein and a blood sample was collected into *EDTA* tubes and *stored* at –20°C for future molecular studies. Abdominal sonography was the standard screening procedure and performed in dorsal or lateral recumbency using a high-definition ultrasound system. The kidneys and bladder were examined using B-Mode. The kidneys were examined for presence of hydronephrosis or proximal hydroureter. The bladder was examined and the location of the ureterovesical junctions was searched. In some dogs, the small colliculi that form the ureterovesical junction were well visible in the trigone area. If possible, visible jets of urine through the ureteral openings were documented separately in longitudinal and transverse planes in B-Mode[22], Color Doppler or Color B-flow mode. An effort was made, to visualize the ureteral openings and the vesicourethral junction together in order to be able to judge the distance between the two structures. Intravenous crystalloid infusion (lactated Ringer's solution) at a rate of 10ml/kg BW and furosemide at 1mg/kg BW IV were added in case the jet phenomenon could not or be only insufficiently demonstrated. This was always done after examination of the kidneys to avoid overinterpretation of iatrogenic distension of the renal pelves after IV fluid administration. If the ureteral openings could not be convincingly localized and/or separately recorded, sonography was followed by contrast-enhanced computed tomography (CT excretory urography) or urethro-cystoscopy provided the owner's consent for general anesthesia and the examination was given.

The proximal boundary of the urethra was defined as the point distal to the bladder, from where the diameter of the urinary tract remained constant.[23] Administering soft manual pressure on the abdomen during sonography assisted in distinguishing between bladder neck (which dilates) and urethra (whose walls stay parallel). In males, the cranial border of the prostate defines the beginning of the proximal urethra. A normal distance between the ureteral openings and the vesicourethral junction was expected to be at least 1.5 to 2cm dependent on the size of the dog. Lesser distances were considered to be ureteral orifice terminations in the "bladder neck".

Surgery or pathology reports were included if sufficiently conclusive. Eight of these cases with reports from before 2008 were included in the dataset. CT scans performed at any other institute for the purpose of EU identification were also recognized. Every written exam-report was reviewed by the same individuals in Zurich and classified according to the following

system: Normal, if the ureteral orifice terminated at the "bladder trigone"; ectopic intravesicular, if it terminated at the "bladder neck" and ectopic extravesicular, if it terminated at the "urethra", which also included openings just at the vesicourethral junction. According to the more caudal location of the right or left ureteral orifice, each dog was assigned one of four phenotypes: phenotype trigone, phenotype IVEU, phenotype EVEU or unknown phenotype.

Statistical analysis— Descriptive statistics were calculated for age at diagnosis, gender, year of birth, inbreeding coefficient, litter size, sex ratio, early-death and season. The inbreeding coefficient of every EMD examined was calculated[d] using a large pedigree[e] of 14'950 dogs with 53 founders and births dating back to 1924. Litter size was defined as the total number of puppies born in a litter including stillborn pups. Sex ratio was calculated as the number of male puppies divided by the total number of pups per litter. The variable early-death includes stillborn littermates or puppies that died during their first 8 weeks of life for various reasons. Information on litter size, sex ratio, number of early-death pups and their gender was only available for Swiss and German EMDs, so any calculations concerning these parameters were carried out with these dogs only. Season specifies the time of the year in which each dog was born. Season 1 was defined as a births between 1st December and the last day of February, Season 2 between 1st March and 31st May, Season 3 between 1st June and 31st August and Season 4 between 1st September and 30th November. In order to evaluate the consequences of breeding restriction, EMDs born in 2006/2007 were compared to those born in 2009/2010 with regard to the selection criteria applied. We distinguished between the offspring of one kennel club that strictly excluded all EVEU phenotypes from breeding and all other kennel clubs where exclusion was voluntary.

Categorical variables such as gender, year of birth, early-death and season were analyzed using contingency tables. Continuous variables such as age at diagnosis, litter size, inbreeding coefficient and sex ratio were checked for normality using QQ plots. If normal distribution was not fulfilled, the appropriate non-parametric tests were performed. Gender distribution of early-death puppies within the litters was evaluated against a ratio of 1:1. Additionally, an expanded dataset of the last 12 years (2000-2011) for gender of all born and all early-death puppies was examined.

After a preliminary univariate analysis of all variables, a multivariate logistic regression was performed to determine associated risk factors. The three phenotypes were encoded differently in binary logistic regression to examine influences on various phenotype groupings: EU-affected (phenotype trigone vs. IVEU and EVEU phenotypes), IVEU (phenotype IVEU vs. trigone and EVEU phenotypes) and EVEU (phenotype EVEU vs. trigone and IVEU phenotypes).

A mixed logistic regression model was also examined that accounted for possible correlated outcomes of littermates, in case the standard logistic model was too conservative. The categorical variables considered in the multivariable regression models were gender, early-death, season. The continuous variables were inbreeding coefficient, litter size and sex ratio. Potential interactions e.g. inbreeding coefficient and litter size, gender and sex ratio were also evaluated. As dogs of the same litter have the same inbreeding coefficients, litter size, sex ratio and early-death information, litters were incorporated into the model as a random factor. Each variable was added separately to the model and its effect evaluated on the basis of AIC (Akaike Information Criterion).[24] The lowest AIC indicates the best fitted model. Log odds were used to measure the association between each categorical variable and phenotype groups.

Descriptive statistics were performed with standard software,[f] whereas for the mixed linear models another software package was used.[g] Descriptive results are presented as median values with range given in parenthesis. Values of $P < 0.05$ were considered significant.

Segregation analysis— Complex segregation analysis of the EU trait was carried out using the Pedigree Analysis Package (PAP).[h] EU was encoded either as a dichotomous or as a trichotomous trait. The following combinations were evaluated separately: Trichotomous dataset 1 (phenotype trigone as unaffected, phenotype IVEU as affected and phenotype EVEU as more severely affected); dichotomous dataset 2 (phenotype trigone as unaffected and phenotypes IVEU and EVEU as affected); dichotomous dataset 3 (phenotypes trigone and IVEU as unaffected and phenotype EVEU as affected); dichotomous datasets 4 and 5 (phenotype trigone as unaffected and phenotype EVEU or IVEU as affected with corresponding phenotype IVEU or EVEU encoded as 0).
Besides family and phenotype input, PAP requires prevalence information on phenotypes. The prevalence used was based on our screening results and calculated for the different phenotype groupings within the datasets.
Maximum likelihood procedures were used to estimate the following parameters: frequency of allele A; the probabilities for transmitting allele A for the three genotypes AA, Aa and aa; the dominance effect of allele A; displacement, which is the distance of the genotypes AA and aa on the liability scale and heritability of the disease.
Five models were evaluated. First a general genetic model which allows free segregation of alleles giving estimates of all 7 parameters. The second was an environmental model which excludes all genetic effects by setting the three transmission probabilities to be equal and keeping heritability at 0. The third was a mixed inheritance model where transmission probabilities are fixed at 1, 0.5 and 0 according to the Mendelian expectation. The mixed model allows the presence of a major gene with a polygenic background. The fourth model was a major gene model which is distinguished from the mixed inheritance by keeping heritability at 0. The fifth was a polygenic model where only heritability is estimated.
Only nested models were compared to each other based on the difference between their likelihoods (-2 ln L), which follows a χ^2 distribution with degrees of freedom equal to the difference in the number of the parameters estimated. A difference between two models was considered significant if $P \leq 0.05$. In case of significant differences between models, the tested model is rejected, whereas with an insignificant difference, the model with fewer parameters is superior in explaining the data. This allows for comparison of the environmental with the general genetic model, the mixed inheritance with the general genetic model, and the major gene and polygene model with the mixed inheritance model.

Results

From 10 European countries 565 EMDs (288 females, 277 males) belonging to 360 different litters out of 161 different kennels were clinically investigated. Median age at diagnosis was 2.4 years (1.8 months - 13.4 years).
Altogether, 550 abdominal sonographic screenings were carried out, of which 37 were not conclusive. Thirty-five CT scans and 7 urethro-cystoscopies were performed, but in 1 CT scan the phenotype could not be defined. In one dog the ureteral openings were only localized during surgery, and in one at necropsy.
Classification into one of the three phenotypes (trigone, IVEU, EVEU) was possible in 552 dogs, and in 13 individuals the phenotype could not be determined. One third (32.9%) had normal terminations of both ureters in the bladder trigone area, 47.3% had at least one IVEU and 19.8% had at least one EVEU termination (Table 1). There was a significant difference in gender distribution with more males being affected than females ($P < 0.001$).
Urinary incontinence was noted in 40 dogs. In 9 animals the incontinence status remained unknown and one incontinent EMD had no phenotype associated. Of all IVEU phenotypes 3% (6 females, 4 males) and of all EVEU phenotypes 27% (23 males, 7 females) were incontinent. Permanent incontinence was present in 4 males and 1 female of the EVEU phenotype, all others were intermittently incontinent. At the time of examination 30

incontinent dogs were intact which also included all permanently incontinent dogs. Eight bitches were spayed and in two males the neutering status could not be assessed retrospectively. Hydronephrosis and/or hydroureter were observed in 11 females and 7 males. Fifteen of which were diagnosed with at least one extravesicular opening and 3 had ectopic intravesicular openings. Eight dogs showed no apparent clinical signs, whereas the others were incontinent (n = 7) or showed abdominal distention or pain (n = 3).

In the year of 2008, a decline of EVEU affected animals became evident (Table 1). There was a significant change in the distribution of phenotypes when comparing dogs born in the two years before the screening started with dogs born in the first two years after breeding restrictions had been established ($P = 0.001$). A marked decline in phenotype EVEU became evident after strict breeding restrictions ($P < 0.001$) but not after voluntary breeding restrictions ($P = 0.142$; Table 2). The inbreeding coefficient could be calculated for 541 phenotyped EMDs with a median of 0.3963 (0.20 - 0.44). Median inbreeding coefficients of each of the three phenotypes were comparable when taking into account all EMDs, or when considering dogs born before (2006 and 2007, n = 132) or after (2009 and 2010, n = 147) breeding restrictions were implemented in 2009 ($P = 0.503$, $P = 0.860$ and $P = 0.312$, respectively; Table 3).

Data from 430 Swiss and German EMDs belonging to one of the three phenotypes (trigone, IVEU and EVEU) was analyzed by logistic regression. Animals were born from 273 litters with a median litter size of 6 (1 - 11) and a median sex ratio of 0.5 (0 - 1). Of all 1543 puppies in those litters 63 died early. No difference in gender distribution was found within the early-death puppies ($P = 0.450$). The same was true when all puppies or only the ones that died early within the last 12 years were considered (n = 3820, $P = 0.116$ vs. n = 274, $P = 0.589$). Most of the EMDs examined were born in season 2 (n = 154) followed by season 3, 4 and 1 (n = 124, n = 87 and n = 65, respectively). There was weak evidence of an association between season and phenotype EVEU ($P = 0.060$) with overrepresentation of phenotype EVEU in season 3. This association existed also when analyzing seasonal influence on phenotype in all EMDs ($P = 0.053$). However, season was not significantly associated with the occurrence of EUs in the logistic regression models. When analyzing groupings of EU-affected and EVEU, the best-fit model revealed gender as a supporting covariate with AIC of 497 and 418, respectively, whereas all other models had poorer AIC. No improvement was achieved when adding interactions. The log odds in these models for the male gender were 1.775 and -0.727, respectively, and female gender decreased this log odds ratio by 1.58 and 1.676. With the phenotype grouping IVEU the best-fit model revealed no variable associated with IVEU.

In the complex segregation analysis 572 individuals were employed, 282 of them (141 male, 141 female) had their phenotype determined. Data consisted of 18 full litters, with phenotypes in all living puppies (n = 56) and both parents identified (litter size range 1 - 8, mean litter size 3.3) and 43 litters with no more than 50% missing members (litter size range 1 - 9, mean litter size 4.6, total of 127 puppies). Pedigree information on an additional 290 animals (siblings and ancestors) with unknown phenotype was also included to connect families. Seven discrete families were formed out of all these individuals in order to reduce the number of inbreeding loops. In dataset 4 and 5 pedigrees had to be adjusted and litters with only phenotype IVEU or EVEU puppies excluded (Table 4).

The 5 datasets which resulted from different phenotype groupings were evaluated for the 5 hypothesized transmission models. They were analyzed in autosomal mode using our reported prevalences (Table 4). The analysis of dataset 1, 2, 3, and 4 resulted in the rejection of the environmental model. In each of these, the mixed inheritance model was superior to the general genetic model, but no difference between mixed inheritance, major and polygene model was detected in dataset 1, 2 and 3. In dataset 4, the polygene model was rejected while the major gene model was superior to the mixed inheritance model. In dataset 5, the

environmental model fitted the data better than the general genetic model. Analysis of dataset 1, 2 and 3 in X-linked mode was attempted, but no parameter estimates were achieved (results not shown).

Discussion

This is the first report to demonstrate a hereditary basis for EUs in dogs that had been suspected for decades. In 1974 Hayes already suggested a genetic involvement with breed predispositions.[9] An increased incidence of EUs in the EMD has been reported previously[a,b] and "based on the high frequency of incontinent dogs in the breed, occurrence of the disorder in littermates, and evidence of parent-to-offspring transmission" North suspected the Entlebucher urinary syndrome (representing primarily EUs) to be hereditary.[2] Using complex segregation analysis in 552 EMDs we could clearly prove the hereditary basis of the disease since the environmental model was rejected in all datasets except for one. In these, the models based on Mendelian expectation of segregation were superior to the general genetic model which allows for free segregation. However, our data and the phenotype grouping presented here do not allow clear differentiation between mixed inheritance, major and polygene models. When IVEU phenotypes were excluded from the dataset by grouping them as unknown, the major gene model fitted best. The presence of a major gene responsible for the disease seems likely and was already suspected by North, who found a high incidence of EU in the EMD.[2] The major gene theory is also supported by the fact that the implementation of a breeding restriction policy of affected dogs greatly decreased the prevalence of EVEU, which cannot be explained only by polygenic inheritance pattern.

The results showing the high prevalence of affected dogs in the population presented here are in accordance with our previous study.[c] We found that only one third of the animals had normal ureteral terminations in the bladder trigone area and that 47% had IVEUs and 20% had EVEUs. The small population size of the breed in conjunction with the high degree of inbreeding might have contributed to this alarming situation.[e]

The possibility of imposing further restrictions has been critically discussed. With breeding constraints already in use due to other disease conditions (hip dysplasia, progressive retina atrophy), the degree of inbreeding and thus danger to the health status of the population might increase. However, the results of the strict breeding restrictions did not show a negative influence on inbreeding coefficients, and the decline of EVEUs from 20% to 1.5% within a relatively short period of time is encouraging. This might reflect the awareness of breeders towards the problem and their readiness to travel larger distances for matings. The high percentage of clinically affected dogs was probably supportive of a more careful breeding selection process. In our study 7% of EMDs showed urinary incontinence and 3% had hydronephrosis and/or hydroureter. Urinary incontinence, which is an important clinical condition is often underestimated, although it may greatly compromise the physical well-being of the patient and can, if not treated successfully, be a reason for euthanasia. Surgical treatment with neoureterostomy, neouretercystotomy or cystoscope-guided laser ablation is expensive. The success rate of achieving urinary continence postoperatively is reportedly 37-72%.[3-5,12,14,25] Urinary tract infections, ascending pyelonephritis and chronic kidney failure are associated conditions after surgery.[3,13,14]

The variable clinical presentation of dogs with EUs along with a high degree of morphological differences in forms of IVEUs or EVEUs, intramural or extramural, unilateral or bilateral and whether a ureterocele, hydronephrosis and/or hydroureters are present, make accurate phenotyping very difficult.[2] We considered contrast-enhanced computed tomography (CT) as the gold standard of imaging modalities for diagnosing EUs, as it is more useful than other established imaging techniques for determining the site of abnormal ureteral termination.[26] Furthermore, with CT, the morphology of the upper urinary tract can also be evaluated, which is not possible with urethro-cystoscopy, a suitable method for the

localization of the ureteral openings. Both of these methods require general anesthesia rendering them less practical for population screenings.[i] Even though ureteral troughs, branches, additional openings and vaginal openings cannot always be detected by sonography we chose this approach for population screening because of its practicality. The procedure may be performed within reasonable time, there is no need for general anesthesia, and it is affordable for owners. Renal morphology can be evaluated using B-mode, and ureteral jets localized by Color Doppler or Color B-flow sonography.[a,i] On the other hand, it requires a lot of practice, the quality of the examination is highly operator dependent, and it may not always provide explicit results. Administration of intravenous fluids and furosemide improves the visibility of the jets considerably if they are poorly seen.[27] Application of soft abdominal pressure to compress the bladder can improve visibility and distinction of the bladder neck, although it still remains somewhat subjective. Apart from occasional limited visibility, a further drawback is the lack of anatomical landmarks. While the internal urethral orifice is clearly visible in most dogs, identification can be hard in others. It is even more delicate to correctly locate the trigone area. As development of the trigone depends, at least in part on intercalation of ureteral and bladder musculature,[28] the trigone area is malformed in dogs with EUs making classification challenging. Also it is possible that there is a wide range of "normal" openings with no effect on functionality, which might have led us to overestimate the IVEU phenotype. However, in the previous comparative study of Entlebucher with Appenzeller Mountain Dogs, a related breed of similar size, this "wide range" was only seen in EMDs.[c] The clinical relevance of the IVEU phenotype is also supported by the fact that 10 of the 262 EMDs with IVEUs showed urinary incontinence and 3 had hydronephrosis and/or hydroureter, thus it cannot be neglected. Without an exact definition of normal openings in the trigone as opposed to IVEU openings, the diagnosis relies on subjective judgment, representing the biggest limitation of this study. The distance between the ureterovesical junction and the internal urethral orifice was almost always measureable and helped with distinction. Even though up to now no normal values have been established by sonography, based on our experience, a distance of 1.5 to 2cm can be considered normal depending on the size of the dog. This is in agreement with data from dogs evaluated by CT, where distances of 1.8 to 3.9 cm were reported.[23] Although this measurement was found to be independent of bladder filling,[23] owners were still asked to withhold their dogs from urinating for three hours before examination as a full bladder helps sonographic examination. In a previous study, an inter-ureteral distance of less than 4mm in urethro-cystoscopy was used to define IVEUs.[2] Using this approach only bilateral IVEUs will be detected, furthermore, this distance is dependent on bladder filling.[23] It is not suitable for screenings without general anesthesia to standardize bladder volume, and therefore we did not use this approach. CT was chosen most often as a backup diagnostic procedure because of its noninvasiveness and reliability.[23,26] A comparison of sonography and CT to diagnose EUs has not been published yet. Our preliminary findings on CT and necroscopy measurements compared with sonography indicate good consistency.

We accounted for the debatable identification of the IVEU phenotype in the complex segregation analysis by grouping it as either abnormal, normal or no information in the different datasets. Dataset 1 assessed the possibility of a gradual relationship between phenotypes trigone, IVEU and EVEU. However, up to now, no genetic or embryologic data supports a common background of origin for IVEUs and EVEUs. In dataset 2, phenotype IVEU is grouped with EVEU accounting for a common genetic background, but it also relies on an accurate differentiation between normal and abnormal which, as mentioned, is problematic. In dataset 3, phenotype IVEU is grouped as normal thus considering the possibility that phenotype EVEU is genetically independent of IVEU without interaction. This excludes a possible common background and is inconsistent with clinical findings of affected dogs in both groups. Dataset 4 groups phenotype IVEU as no information and is

based on a similar independence between phenotype IVEU and EVEU but without making assumptions on the former. Unfortunately, this is associated with a loss of data. However, the authors consider this approach the best fitting for several reasons. First the phenotype which is the hardest to accurately diagnose (IVEU) is excluded while keeping the other, more reliably identifiable phenotypes in. Second those included are also the clinically more relevant phenotypes. Third an interaction between the IVEU and EVEU phenotypes in the form of a polygenic background is not excluded. For completeness, EVEU phenotypes were similarly excluded in dataset 5. Using this dataset, information on the clinically more severely affected and therefore most interesting group is lost and the distinction of phenotype groups is inaccurate. When comparing results from the different datasets accounting for their limitations and taking our clinical results into account, a major gene inheritance associated with the EVEU phenotype on a polygenic background for EUs in general seems likely. The documented incidence decline of mainly EVEU phenotypes supports this assertion.

In order to assess possible risk factors associated with EUs, several parameters were statistically evaluated with logistic regression. Of all the variables only gender was proven to have an influence on the two phenotype groupings, EU-affected and EVEU but not on IVEU. Females had a lower chance of being EU-affected or having EVEU than males. The finding of a possible association with gender warranted the evaluation of an X-linked mode, but our dataset was not sufficient to prove or exclude an X-linked mode of inheritance. An extended dataset with improved family structure would be necessary to ascertain gender influence. At the moment we cannot exclude that one of the genes located on the X-chromosome does play a role. This needs to be verified with molecular genetic studies in the future. Although involvement of an X-linked factor cannot be completely ruled out, critical evaluation of gender distribution is indicated. Affected females that have been lost early on due to resorption of embryos, fetal or neonatal death could be a possible explanation to our findings. However, the ratio of female and male EMDs born in the German and Swiss populations in the last 12 years was similar. Gender distribution of early-death puppies was not significantly different either. Bias could have arisen during diagnostic work-up if males were more strictly classified than females. Historically, more females are reported to have the disease[12] although the true prevalence in males must have been underestimated.[14,29] Using the prostate as a landmark in males facilitates the classification of EVEU. In older intact male dogs benign prostatic hyperplasia has to be taken into account, because a cranially enlarged prostate can compress the bladder neck, which may then be mistaken for the urethra. During the screening process we focused on the junction of the prostate and the urethra, which concurs anatomically with the openings of the deferent ducts. Still, this could have biased our assessment in males. On the other hand, urinary incontinence associated with EUs is more common in males than in females, further supporting our findings of a higher prevalence in males.

The frequency of EU phenotypes as well as of clinically affected dogs reported here might not reflect the true prevalences in the breed. At the beginning of our screening study, clinically apparent EMDs and dogs from affected families were more likely to be presented for examination. Additionally, in order to get a more complete picture on the families, owners of dogs of affected littermates were requested to come for screening. The above mentioned presentation of cases and thus the composition of our study population, could have led to an overestimation of clinically affected or EVEU phenotype dogs. Additionally, 8 cases from before the start of the screening program were also included and all of them were grouped as EVEU. Since 2008 the screening evaluation has become obligatory for all breeding dogs in Germany, so the selection of cases that might have been biased in the beginning has diminished over time.

In the current study we were able to demonstrate a hereditary basis of EUs in dogs by example of the Entlebucher Mountain Dog. The complex inheritance pattern of the disease

likely involves several genes as well as a major gene in association with the clinically more relevant EVEU phenotype. The EMD breed seems to be highly affected both clinically and phenotypically. This may allow us to identify molecular markers that will help to understand the genetic base and pathogenesis of the disease in other dog breeds and in humans as well. Fortunately the incidence of EU has already decreased considerably in the EMD breed shortly after breeding restrictions have been implemented. In the future, restriction policies should be carefully planned and orchestrated between kennel clubs to ensure the health of the breed.

Footnotes

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Tables

Table 1— Phenotype, year of birth and gender distribution of 565 Entlebucher Mountain Dogs examined for ectopic ureters.

Year of birth	Total Male/Female	Phenotype trigone Male/Female	Phenotype IVEU Male/Female	Phenotype EVEU Male/Female	Phenotype unknown Male/Female
Before 2002	40 27/13	12 7/5	8 2/6	17 16/1	3 2/1
2002	24 14/10	4 1/3	11 6/5	8 6/2	1 1/0
2003	28 12/16	9 1/8	12 7/5	7 4/3	0
2004	47 19/28	16 6/10	22 7/15	6 4/2	3 2/1
2005	48 26/22	18 8/10	19 10/9	11 8/3	0
2006	73 36/37	19 3/16	37 19/18	17 14/3	0
2007	63 36/27	15 5/10	29 18/11	17 13/4	2 0/2
2008	86 37/49	27 7/20	45 21/24	10 7/3	4 2/2
2009	93 45/48	32 9/23	50 29/21	11 7/4	0
2010	55 19/36	27 7/20	24 8/16	4 4/0	0
2011	8 6/2	2 0/2	5 5/0	1 1/0	0
Total	565 277/288	181 54/127	262 132/130	109 84/25	13 7/6

Phenotype IVEU = Termination of the most caudal ureteral opening is intravesically ectopic.
Phenotype EVEU = Termination of at least one ureteral opening is extravesically ectopic.

Table 2—Phenotypes of ectopic ureters in Entlebucher Mountain Dogs born before (2006 - 2007) and after (2009 - 2010) breeding restrictions established by kennel clubs in 2009. Results are shown for all clubs before and after, as well as separately for those employing voluntary vs. strict restrictions.

		Phenotype trigone	Phenotype IVEU	Phenotype EVEU
Breeding restriction				
Born before	All	34 (25.4%)	66 (49.2%)	34 (25.4%)
Born after	All	59 (39.9%)	74 (50.0%)	15 (10.1%)
	Voluntary	69 (83.1%)		14 (16.8%)
	Strict	64 (98.5%)		1 (1.5%)

Phenotype IVEU = Termination of the most caudal ureteral opening is intravesically ectopic.
Phenotype EVEU = Termination of at least one ureteral opening is extravesically ectopic.

Table 3— Mean of inbreeding coefficients with range given in parenthesis according to ectopic ureter phenotypes for all Entlebucher Mountain Dogs examined, and for those born before (2006 - 2007) or after (2009 - 2010) breeding restrictions were established by kennel clubs in 2009.

Inbreeding coefficient	Phenotype trigone	Phenotype IVEU	Phenotype EVEU
All (n = 541)	0.3958 (0.2-0.43)	0.3973 (0.2-0.44)	0.3951 (0.37-0.43)
Breeding restrictions			
Born before (n = 132)	0.3917 (0.36-0.42)	0.3933 (0.29-0.42)	0.3939 (0.37-0.43)
Born after (n = 147)	0.3999 (0.2-0.42)	0.4004 (0.20-0.43)	0.4101 (0.39-0.42)

Phenotype IVEU = Termination of the most caudal ureteral opening is intravesically ectopic.
Phenotype EVEU = Termination of at least one ureteral opening is extravesically ectopic.

Table 4— Segregation analysis of 5 datasets resulting from different phenotype groupings for ectopic ureters in Entlebucher mountain Dogs.

Dataset	Prevalence	Animals	Model comparison	χ^2	df	P-value
1	Trigone 0.33 IVEU 0.47 EVEU 0.20	Total 572 Phenotyped 282	General genetic - Environmental	30.026	4	< 0.001
			- Mixed inheritance	3.308	3	0.347
			- Major gene	0.861	1	0.353
			- Polygene	5.222	3	0.100
2	Trigone 0.33 IVEU and EVEU: 0.67	Total 572 Phenotyped 282	General genetic - Environmental	11.947	4	0.018
			- Mixed inheritance	2.283	3	0.516
			- Major gene	0.199	1	0.656
			- Polygene	1.897	3	0.594
3	Trigone and IVEU 0.80 EVEU: 0.20	Total 572 Phenotyped 282	General genetic - Environmental	21.390	4	< 0.001
			- Mixed inheritance	1.755	3	0.625
			- Major gene	2.911	1	0.088
			- Polygene	5.534	3	0.130
4	Trigone 0.80 EVEU: 0.20	Total 451 Phenotyped 137	General genetic - Environmental	26.012	4	< 0.001
			- Mixed inheritance	4.530	3	0.210
			- Major gene	0.452	1	0.501
			- Polygene	20.517	3	< 0.001
5	Trigone 0.53 IVEU 0.47	Total 540 Phenotyped 216	General genetic - Environmental	9.252	4	0.055
			- Mixed inheritance	19.633	3	< 0.001

χ^2 = chi square value, equal to the difference of the model tested with the general genetic model (for environmental and mixed inheritance model) or of the tested model with the mixed inheritance model (for the major gene and polygene model).
df = degrees of freedom, equal to the difference in estimated parameters between compared model

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